Activation of the annexin 1 counter-regulatory circuit affords protection in the mouse brain microcirculation

Felicity N. E. Gavins,* Jesmond Dalli,* Roderick J. Flower,* D. Neil Granger,† and Mauro Perretti*,[1]

*William Harvey Research Institute, Charterhouse Square, London, UK, and †Louisiana State University Medical School, Shreveport, Louisiana, USA

ABSTRACT **The purpose of this study was to investigate the role of the homeostatic antiinflammatory axis centered on annexin 1 (AnxA1) in cerebral microvascular dysfunction and tissue injury associated with middle cerebral artery (MCA) occlusion and reperfusion. Intravital fluorescence microscopy was used to visualize the mouse cerebral microcirculation: AnxA1 null mice exhibited more white blood cell adhesion in cerebral venules than their wild-type counterparts, and this was accompanied by a larger cerebral infarct vol and worse neurological score. All parameters were rescued by delivery of human recombinant AnxA1. To further explore these findings using pharmacological tools, the effect of a short AnxA1 peptidomimetic was tested. When given during the reperfusion phase, peptide Ac2–26 produced similar cerebroprotection, which was associated with a marked attenuation of cell adhesion and markers of inflammation as measured in tissue homogenates. The pharmacological effects of peptide Ac2–26 occurred *via* receptors of the formyl-peptide receptor (FPR) family, most likely FPR-rs2, as deduced by displacement assays with transfected cells and *in vivo* experiments with transgenic mice and receptor antagonists. Our findings indicate that the endogenous antiinflammatory circuit centered on AnxA1 produces significant cerebral protection, and that these properties might have therapeutic potential for stroke treatment.—Gavins, F. N. E., Dalli, J., Flower, R. J., Granger, D. N., Perretti, M. Activation of the annexin 1 counter-regulatory circuit affords protection in the mouse brain microcirculation *FASEB J.* 21, 1751–1758 (2007)**

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THE ROLE OF INFLAMMATORY MECHANISMS in pathologies previously believed to be independent from the host response has recently been appreciated, and it is now clear that inflammation contributes to the pathogenesis of many cardiovascular diseases, including stroke. Stroke consists of an irreversible neurological deficit that may be produced by inadequate perfusion of the brain or brain stem (1); in large part it is ischemic in origin, with a smaller incidence (~15%) of hemorrhagic stroke. Therapeutic intervention is based on restoring blood flow either by supplying volumes (hemorrhagic stroke) or by dissolving the thrombus with tissue plasminogen activator (tPA): thus, in treatment of stroke a phase of reperfusion may occur.

Both focal and global ischemia are associated with inflammation and characterized by rapid vascular responses that are contributory to changes in brain parenchyma that lead to neuronal death. However, whether inflammation postcerebral ischemia is a positive or negative outcome remains inconclusive. Although animal models of stroke have permitted biochemical and physiological measurement of ischemic brain, the relevance to human cerebrovascular disease is controversial and thus could be behind the failure of antiinflammatory therapy trials for stroke, *e.g.*, the humanized anti-CD54 monoclonal antibody (mAb) (2). When blood flow is restored after an ischemic episode, several cascades of events are initiated, producing activation of blood-borne cells and of resident cells (3, 4). In the case of stroke, generation of cytokines and lipid mediators, with the ensuing accumulation of leukocytes (5) as well as the activation of resident glial cells (6) occurs; altogether, inflammatory processes contribute to the tissue damage associated with reperfusion injury (4, 7).

We and others have recently shed some light on biochemical pathways centered on endogenous inhibitors endowed with counter-regulatory and protective functions. They have mostly been studied in the context of acute inflammation and comprise antiinflammatory pathways that operate in parallel, and sometimes in a time-delayed fashion, to the more widely studied proinflammatory mediators, to ensure rapid resolution of the host response with return to tissue homeostasis (8, 9). Our focus has been the glucocorticoid-regulated protein annexin 1 (AnxA1; 346 aa long; 37 kDa protein), a potent inhibitor of leukocyte trafficking in acute and chronic inflammation (10, 11), with a particular efficacy on leukocyte/endothelium interactions promoted by ischemia-reperfusion (I/R) procedures (12, 13). Myeloid cells are rich in AnxA1, and on cell activation this 37-kDa protein translocates on the external leaflet of the membrane where it interacts with a specific G-protein coupled receptor, termed formyl-peptide receptor like 1 (FPRL-1) (14). This system has been better dissected for the polymorphonuclear leukocyte and a model for an autocrine/paracrine circuit by

[1]Correspondence: The William Harvey Research Institute, Barts and The London, Charterhouse Sq., London, EC1M 6BQ, UK. E-mail: m.perretti@qmul.ac.uk

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which AnxA1 and its receptor control leukocyte activation and adhesion has been elaborated (10). However, it is likely to be applicable to other cell systems, including macrophages (15) and specialized epithelial cells (16).

Considering *a)* indications that a 188-aa long AnxA1 fragment was protective in a rat model of cerebral ischemia (17); *b)* AnxA1 and its peptidomimetics exert protective actions on brain injury also when produced by toxic activation (18); and, as discussed above, *c)* AnxA1 and peptides can inhibit leukocyte-endothelial cell adhesion, the object of this study was to fully test the potential involvement of the counter-regulatory AnxA1 system in a model of focal ischemic stroke.

MATERIALS AND METHODS

Animals

Male wild-type ($AnxA1^{+/+}$) littermate control and AnxA1 null mice ($AnxA1^{-/-}$) (19) or FPR null mice (backcrossed with C57BL/6 for 6 generations (20) and C57BL/6 mice (WT; purchased from B&K Universal Ltd, Hull, UK) (body wt ~30 g in all cases) were used. Mice were maintained on a standard chow pellet diet with tap water *ad libitum* using a 12 h light/dark cycle, in which the temperature was maintained at 21–23°C. Animal work was performed according to Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986).

In vivo protocols

Middle cerebral artery occlusion and reperfusion

A middle cerebral artery (MCA) ischemia reperfusion protocol was performed as recently reported (5, 21). Briefly, mice were anesthetized and maintained on artificial ventilation. Femoral artery and vein were cannulated to monitor mean arterial blood pressure (MABP), sample arterial blood for blood gas analysis, and for intravenously (i.v.) administration of the rhodamine 6G (0.02% in 200 μl) and Texas red (2.5 mg/ml). The middle cerebral artery (MCA) was occluded using the intraluminal filament method (6 nylon), as described previously in detail (5, 21), using a 1 h occlusion period followed by a period of either 4 h or 24 h of reperfusion. Sham-operated mice were subject to anesthesia and other surgical procedures without MCA occlusion and analyzed 5 h or 25 h after the start of surgery.

The head of each mouse was then fixed in a plastic frame in a sphinx position. The left parietal bone was exposed by a midline skin incision, followed by a craniectomy (diameter: 2.5 mm) with a drill at 1 mm posterior from the bregma and 4 mm lateral from the midline. The dura mater was not cut because the fluorescent-labeled leukocytes were readily visualized. A 12-mm glass coverslip was placed over the craniectomy and the space between the glass and dura mater was filled with artificial cerebrospinal fluid (5, 21).

A Zeiss Axioskop "fibrous sheath" microscope with a mercury lamp was used to observe the pia venules in the cerebral cortex. A Hitachi charge-coupled device color camera (model KPC571; Tokyo, Japan) acquired images that were recorded onto video. One to five randomly selected venular segments, 30–70 μm in diameter and 100 μm long, were observed for each mouse after treatment. White blood cell rolling and leukocyte adhesion (a cell that had remained adherent for 30 s or longer) were assessed in 3–5 venules. These parameters were expressed as the number of cells per square millimeter of the venular surface and calculated from diameter and length, assuming cylindrical shape. Estimates of shear rate or pseudo-shear rate in cerebral venules were obtained by fluorescence microscopy based on image analysis determinations of the maximal velocity of fluorescently labeled red blood cells. Such estimates of shear or pseudo-shear rate in venules are obtained using measurements of venular diameter (Dv) and the maximal velocity of flowing red blood cells (V_{cell}) according to the formulation: shear or pseudo-shear rate = $(V_{cell}/1.6)/Dv \times 8$ (21).

Infarct vol

After a 24 h reperfusion period, mice were killed and brains were immediately removed, placed into 4°C PBS for 15 min; 2-mm coronal sections were then cut with a tissue cutter. The brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride in phosphate buffer at 37°C for 15 min and fixed by immersion in 10% formaldehyde. The stained sections were photographed and the digitized images of each brain section (and the infarcted area) were quantified using a computerized image analysis program (NIH 1.57 Image Software).

Neurological score

The functional consequences of cerebral I/R injury were evaluated by using a five-point neurological deficit score [0, no deficit; 1, failure to extend right paw; 2, circling to the right; 3, falling to the right; and 4, unable to walk spontaneously (5, 21)] and were assessed in a blinded fashion. A maximal score of 4 could be assigned to each experimental animal.

Receptor agonists and drug treatment

Human recombinant AnxA1 and its peptide Ac2–26 (Ac-AMVSEFLKQAWFIENEEQEYVQTVK) were obtained as reported (22), and doses selected from previous studies (12, 23). The same applied to the antagonist Boc2 (N-tbutyloxycarbonyl-Phe-DLeu-Phe-DLeu-Phe; ICN Pharmaceuticals, Basingstoke, UK). Animals were injected with saline (100 μl), human recombinant AnxA1 (1 μg per mouse), tPA (Alteplase; Genentech Inc., San Francisco, CA, USA: 10 mg/kg, 2 mg/ml in saline), peptide Ac2–26 alone (100 μg per mouse, ~33 nmol), Boc2 (10 μg, ~12 nmol) alone, or Boc2 and peptide Ac2–26 at various times in the reperfusion phase.

In vitro protocols

Biochemical determinations

Tissue levels of mouse keratinocyte chemoattractant (KC or CXCL1), interleukin-1β, and monocyte chemoattractant protein-1 (MCP-1 or CCL2) were measured using standard enzyme linked immuno-absorbant assays (Quantikine™ immunoassay kits; R&D Systems, Abingdon, UK).

Selected brain samples were homogenized in a phosphate buffer containing 0.5% hexadecyl trimethylammonium bromide (HTAB) detergent, and the degree of PMN accumulation was measured by comparing myeloperoxidase (MPO) activity in tissue extracts as described (13), using human MPO (Sigma) as standard. Data are reported as U per mg of wet tissue.

To quantify specific mRNA levels, total brain RNA was isolated using spin column according to manufacturer's instructions (RNeasy kit, Qiagen, Crawley, UK). Contaminating DNA was removed on the column prior to elution of RNA, using DNase treatment as per manufacturer's instructions (Qiagen). RNA was reverse-transcribed with 2 μg oligo(dT)15 primer (Promega, Southampton, UK), 10 U avian myeloblastosis virus (AMV) reverse transcriptase, 40 U ribonuclease inhibitor (Promega), and 1.25 mM each deoxyribonucleoside triphosphate (dNTP) for 20 min at 42°C. The resultant cDNA

was used for real-time polymerase chain reaction (PCR). An equal amount of first-strand cDNA was amplified by PCR using platinum *Taq*DNA polymerase (Invitrogen). The forward and reverse primers for mouse *fpr1, fpr-rs1, fpr-rs2* (24) and shown in Supplemental Table 1*S*. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as controls. The PCR conditions were 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 30 s with a final extension of 72°C for 7 min. Finally, brain AnxA1 protein expression in samples from sham operated mice or from mice subjected to MCA occlusion and 24 h reperfusion was determined by Western blotting as reported (13).

Statistical analysis

All values are expressed as mean ± SE of mean, with n of animals per group where stated. Statistical analysis for the intravital microscopy studies was assessed either by Student's t test (2 groups) or by one-way ANOVA followed by Bonferroni post hoc test (more than 2 groups). Differences among groups in *in vitro* experiments were determined by the Mann-Whitney test. In all cases, a probability value of $P < 0.05$ was considered significant.

RESULTS

Protection afforded by endogenous AnxA1

Analyses in brain samples after MCA I/R revealed significant changes for AnxA1 mRNA (data not shown) and protein (**Fig. 1*A***). These changes were functional since deletion of *anxA1* provoked significant acceleration of the macroscopic response in mice subjected to IR (Fig. 1*B*): the higher susceptibility of AnxA1 null *vs.* AnxA1$^{+/+}$ mice was evident as early as 6 h postreperfusion, being more marked at the 18 h time point (**Fig. 1*C***). This phenomenon was reflected in differences in mortality between AnxA1$^{+/+}$ and AnxA1 null mice: the latter group had an incidence of death 24 h postreperfusion of 6 out of 10 mice against a 15% (1 out of 7 mice) value for AnxA1$^{+/+}$ mice ($P<0.05$).

These experiments suggested a protective role of endogenous AnxA1 in this experimental model, justifying further detailed analyses. Intravital microscopy of pia mater vessels using epifluorescence revealed time-dependent induction of leukocyte/endothelium interactions with significant cell rolling and adhesion in AnxA1$^{+/+}$ stroke mice above sham animals at both 4 and 24 h (n=6; $P<0.05$) postreperfusion (**Fig. 2*A***). AnxA1 null mice displayed augmented cell adhesion that reached statistical difference from AnxA1$^{+/+}$ mice at the 24 h time point, with an early (4 h) drop in the degree of cell rolling. No differences in cell rolling or adhesion were observed in sham animals (Fig. 2*A*). No differences among the mouse genotype were detected with respect to the hemodynamic parameters (Supplemental Table 2*S*).

The brain inflammatory response was monitored by measuring pivotal cytokines in the injured tissue (Fig. 2*B*); this revealed markedly elevated levels post-I/R compared to sham mice. Absence of *anxA1* produced again very specific effects, with an exacerbation of the MCP-1 response both at 4 and 24 h, prolongation of KC levels (significantly above those of AnxA1$^{+/+}$ mice only at 24 h post-I/R) and no effect on IL-1β (Fig. 2*B*).

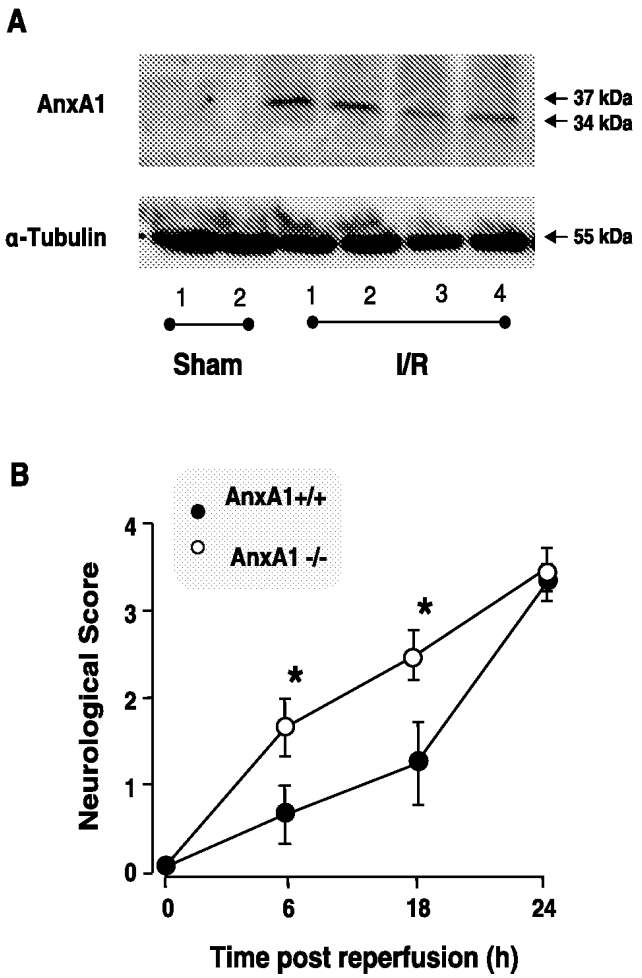

Figure 1. Endogenous AnxA1 and stroke. AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice were subjected to 4 or 24 h reperfusion after 1 h MCA occlusion. *A)* Western blotting analysis of AnxA1$^{+/+}$ brains showing mouse AnxA1 (37 kDa) or α-tubulin (55 kDa) protein expression in 2 (sham) or 4 (24 h post-MCA I/R) distinct brains. *B*) Kinetics of neurological damage (6 mice per group; *$P<0.05$).

Of importance, the large majority of alterations in markers of inflammation and tissue injury displayed by the AnxA1 null mouse were rescued by exogenous administration of AnxA1. **Figure 3** shows a selection of these data as measured 24 h postreperfusion, and similar observations were also made at the 4 h time point (data not shown): for instance, given at the dose of 1 μg per mouse (equivalent to 27 pmol), treatment with AnxA1 restored the phenotype of the AnxA1 null back to the AnxA1$^{+/+}$ mouse phenotype. Finally, we measured MPO activity in brain homogenates as marker of neutrophil infiltration. Figure 3*e* shows data for MPO values, with a marked increase after IR, and an exacerbation in the AnxA1 null mice (U/mg of tissue): 4.3 ± 0.6 *vs.* 6.0 ± 0.5; $n = 6$ $P< 0.05$. Thus this set of experiments revealed tissue-protective effects for exogenous AnxA1, in either genotype, highlighting the pharmacological potential for this antiinflammatory mediator. Based on these results, in the second part of the study the effects of the short AnxA1 derivative, peptide Ac2–26, were tested.

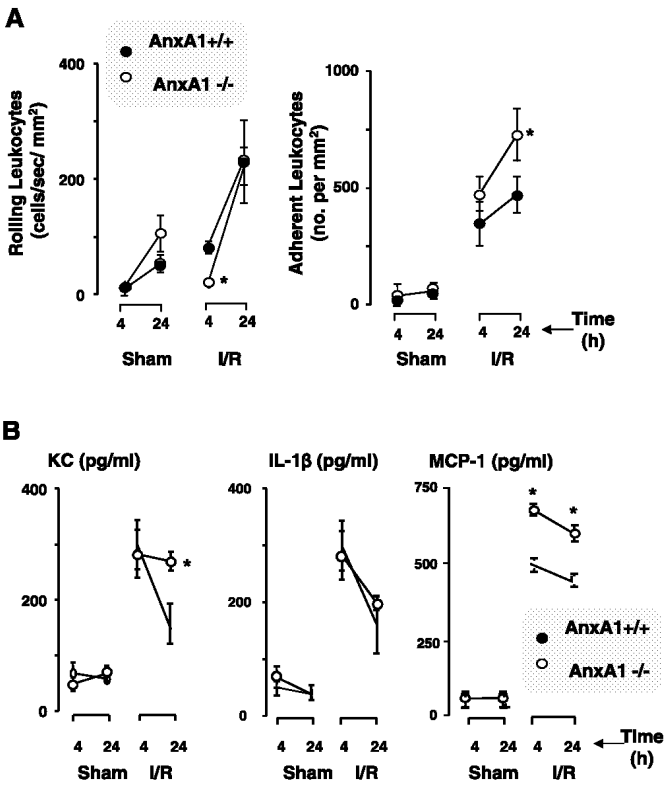

Figure 2. Changes in leukocyte recruitment in AnxA1 null mice. AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice were subjected to 4 or 24 h reperfusion after 1 h MCA occlusion; sham animals were also used. *A)* Cell rolling and adhesion. *B)* Brain KC, IL-1β and MCP-1 levels. Data are mean ± SEM of 6 mice per group. *$P < 0.05$ *vs.* respective AnxA1$^{+/+}$ value.

Effects of peptide Ac2–26

Treatment of mice with 100 μg peptide Ac2–26 significantly reduced the effects of stroke (*e.g.*, 4 h leukocyte adhesion; **Fig. 4*A***). To fully exploit the potential of this AnxA1 derivative for antistroke therapy, the kinetics of peptide Ac2–26 effectiveness was determined at the 24 h time point, where effects on neurological score and infarct vol could be assessed. When administered at time 0 or 6 h postreperfusion, with analyses performed at the 24 h time point, peptide Ac2–26 produced significant yet modest inhibition of leukocyte adhesion to vascular endothelium (Fig. 4*B*) and neurological score (Fig. 4*C*). The most significant inhibitory effects were obtained when peptide Ac2–26 was given at least twice, *i.e.*, at time 0 and 6 h (*e.g.*, cell adhesion down to 27±3 cells/mm^2 n=6; P<0.05), or time 0 and 18 h (*e.g.*, cell adhesion down to 60±11 cells/mm^2 n=6; P<0.05), after MCA occlusion and reperfusion (Fig. 4). These protective actions extended also to a reduction in the 24 h infarct vol (Fig. 4*D*).

The efficacy and reproducibility of the effects of both whole protein AnxA1 and peptide Ac2–26 led us to address, in the final part of the study, the clinical implication of AnxA1 therapy. Data in Supplemental Fig. 1*S* show a parallel between the efficacy of AnxA1 and that of tPA. When administered i.v. over the first 20 min of reperfusion and quantified after 24 h, tPA could attenuate all markers of brain damage, these being neurological score, infarct vol, and cell adhesion on pia mater vessels (Fig. 1*S*).

To support these pharmacological data, we tested the expression of potential receptors responsible for the effects of peptide Ac2–26. Real-time PCR of brain tissue extracts revealed presence of mouse FPR and FPR-rs2, but not FPR-rs1; importantly, MCA I/R augmented FPR-rs2 message (**Fig. 5*A***). We then tested whether the peptide could bind to this receptor, with stably transfected 293-HEK cells using a well-defined binding protocol with radiolabeled peptide Ac2–26 as tracer (14, 25). Cold peptide Ac2–26 efficiently displaced the tracer from FPR with an approximate EC_{50} of 1 μM, as shown in Fig. 5*B*. The same was true when FPRrs2-HEK cells were used (Fig. 5*B*). Of interest, Boc2 could displace tracer binding from either receptor: tested at 1 μM, Boc2 displaced tracer binding to FPR-HEK and FPR-rs2 cells by 60 ± 2% and 73 ± 2%, respectively (mean±SEM of three experiments; P<0.05 *vs.* vehicle).

To dissect the FPR and/or FPRrs2 involvement in the cerebro-protective properties of peptide Ac2–26, experiments were conducted in mice null for FPR. **Figure 6**

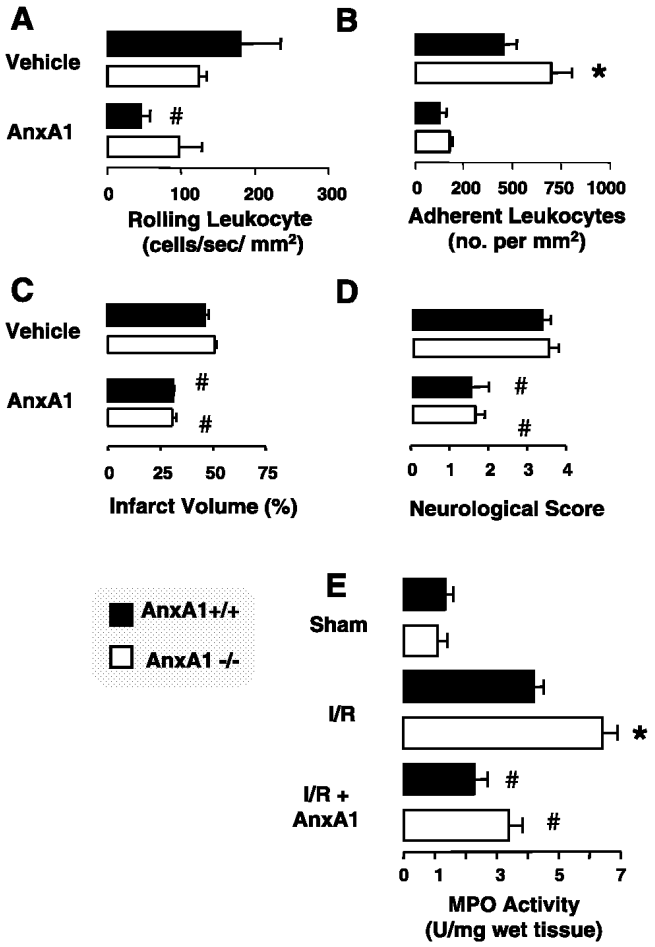

Figure 3. AnxA1 rescues the phenotype of the AnxA1 null mice. *A–D)* Macroscopic and microscopic effects of stroke, as in Figs. 1 and 2; *E)* MPO activity values. In all cases, either vehicle or AnxA1 (1 μg i.v.) were given at time 0, 6, and 18 h after beginning of reperfusion. Data are mean ± SEM of 6 mice per group. *$P < 0.05$ *vs.* respective AnxA1$^{+/+}$ value. #$P < 0.05$ *vs.* respective vehicle value.

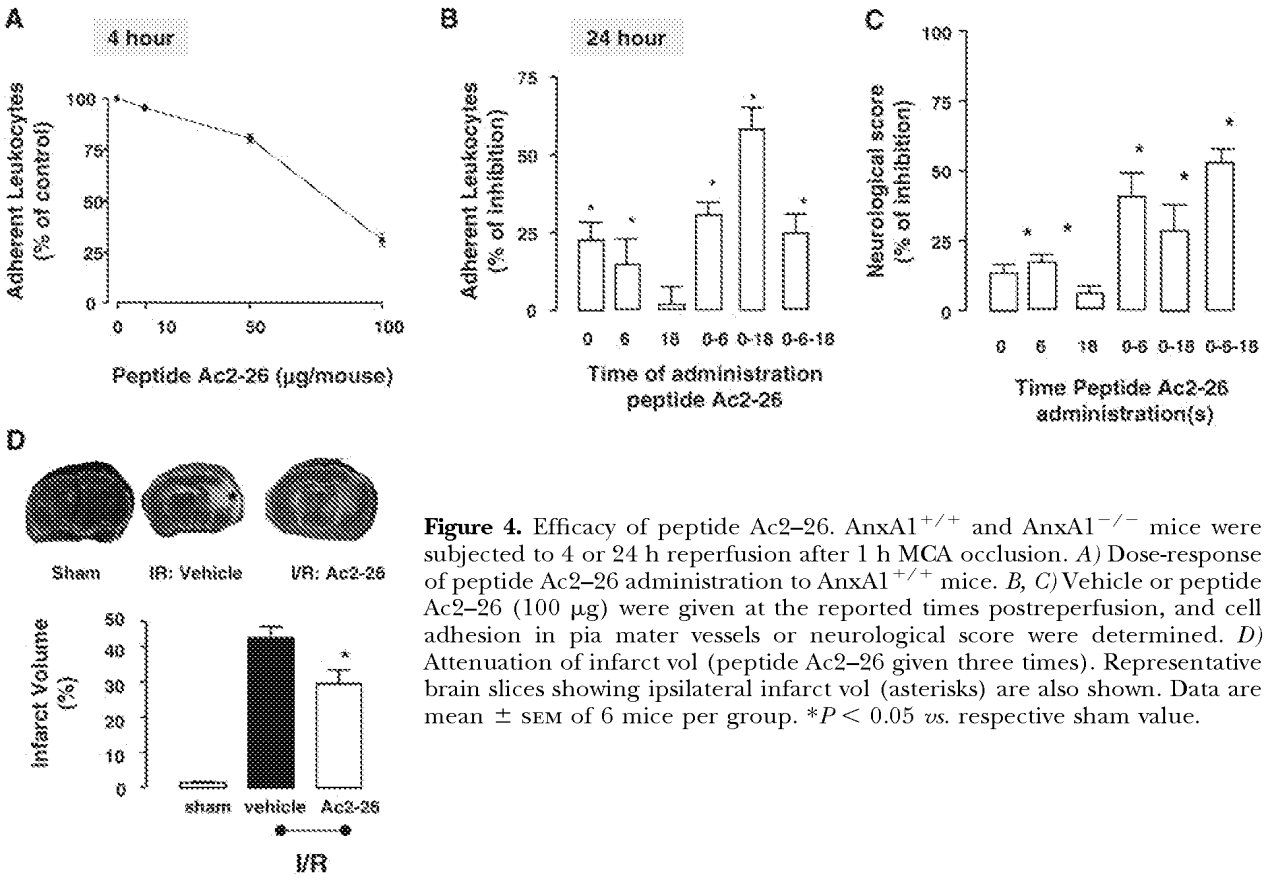

Figure 4. Efficacy of peptide Ac2–26. AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice were subjected to 4 or 24 h reperfusion after 1 h MCA occlusion. *A)* Dose-response of peptide Ac2–26 administration to AnxA1$^{+/+}$ mice. *B, C)* Vehicle or peptide Ac2–26 (100 μg) were given at the reported times postreperfusion, and cell adhesion in pia mater vessels or neurological score were determined. *D)* Attenuation of infarct vol (peptide Ac2–26 given three times). Representative brain slices showing ipsilateral infarct vol (asterisks) are also shown. Data are mean ± SEM of 6 mice per group. $*P < 0.05$ *vs.* respective sham value.

illustrates that the peptide retained its inhibitory actions on the vasculature, producing significant inhibition on cell rolling and cell adhesion (Fig. 6*A*, *B*). The same outcome was obtained when the effect of peptide Ac2–26 on infarct vol was determined (Fig. 6*C*).

Of interest, the pan-antagonist to this family of receptors, Boc2 blocked the actions of the peptide on all parameters under analysis (Fig. 6).

DISCUSSION

In this study we reveal a crucial protective role for the endogenous antiinflammatory and homeostatic mediator AnxA1 in the outcome of focal ischemic stroke, such that we could propose it as a major arm of the innate protective response rapidly activated in stroke (2). This conclusion is substantiated by the exacerbation of the neurological deficit associated with this model in AnxA1 null mice, coupled to augmented vascular responses and inflammatory mediator generation in the brain, as well as the protection afforded by the full-length protein and the pharmacophore bioactive peptide.

Currently, there are not many effective treatment choices for patients affected by ischemic stroke. This pathology affects ~10% of the population, with twice the incidence in the elderly, and the only treatment available is based on the administration of tPA: this agent dissolves the thrombus thereby favoring blood reperfusion and vessel patency. A smaller percentage (~15%) of stroke cases are hemorrhagic in their etiology, yet clinical management entails vol restoration and reperfusion. Research in the past decade or so have highlighted the presence of inflammatory processes in stroke (7). Whereas this fact is now acknowledged, the contribution that blood-borne cells, relative to resident cells (microglia, astrocytes, and neurons), to the pathogenesis of ischemic stroke has yet to be dissected or accepted. This multicellular contribution to stroke may explain the failure of single-target directed strategies, such as antiadhesion molecule therapy where targeting blood-borne cells may leave unaffected the inflammatory component due to resident brain cells.

AnxA1$^{-/-}$ blood cells display altered profiles of activation and are often refractory to regaining a homeostatic proresolving phenotype. For instance, macrophages deficient in the protein are delayed in their activating response to lipopolysaccharide (LPS) but are also unable to subside it (26), a phenomenon partly due to defective receptor recycling and signaling. Similarly, AnxA1$^{-/-}$ neutrophils are more reactive to *in vitro* stimulation than to *in vivo* activation by inflammatory signals in the cremaster microvasculature (27). Thus, marked prolongation of the leukocyte adhesion process was also observed in the brain microcirculation, preceded by a significant delay in promoting cell rolling (reminiscent of the altered profile of inflammation and activation discussed above).

A major outcome of this study is the potential for AnxA1 and its mimetics to attenuate the microvascular

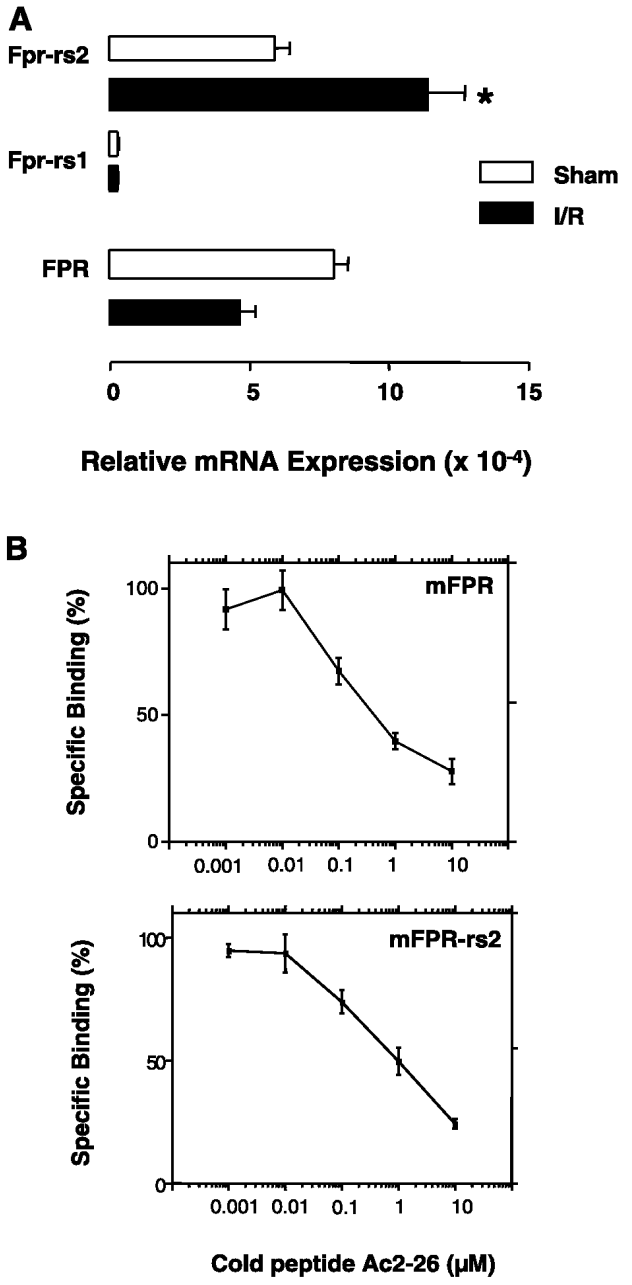

Figure 5. Peptide Ac2–26 binds to FPR-rs2. *A)* Real-time PCR analysis for mouse formyl-peptide receptors mRNA expression in left AnxA1$^{+/+}$ brains. *B)* Displacement curves for cold peptide Ac2–26 on 50 nM [^{125}I-Tyr]-Ac2–26 used as tracer in transfected FPR-rs2-HEK-293 cells. Data are presented as mean ± SEM of 3 independent experiments. *$P < 0.05$ *vs.* respective sham value.

dysfunction and tissue injury associated with ischemic stroke. Administration of small doses of the protein rescued the phenotype of the AnxA1$^{-/-}$ mouse and a significant degree of cerebro-protection was also obtained by dosing the animals with peptide Ac2–26. In the mouse, peptide Ac2–26 has a relatively short half-life <1 h (28)]; nonetheless it produced modest yet significant inhibition of the vascular response after focal stroke even when given once either at time 0 or 6 h into reperfusion, but only at the dose of 100 μg/mouse dose. The latter data together with the more pronounced protection afforded by repeated dosage with peptide Ac2–26 clearly highlights the potential therapeutic implications of our findings in this model of ischemic stroke.

In the human system, peptide Ac2–26 binds to and activates all three receptors of the FPR family (14, 29,

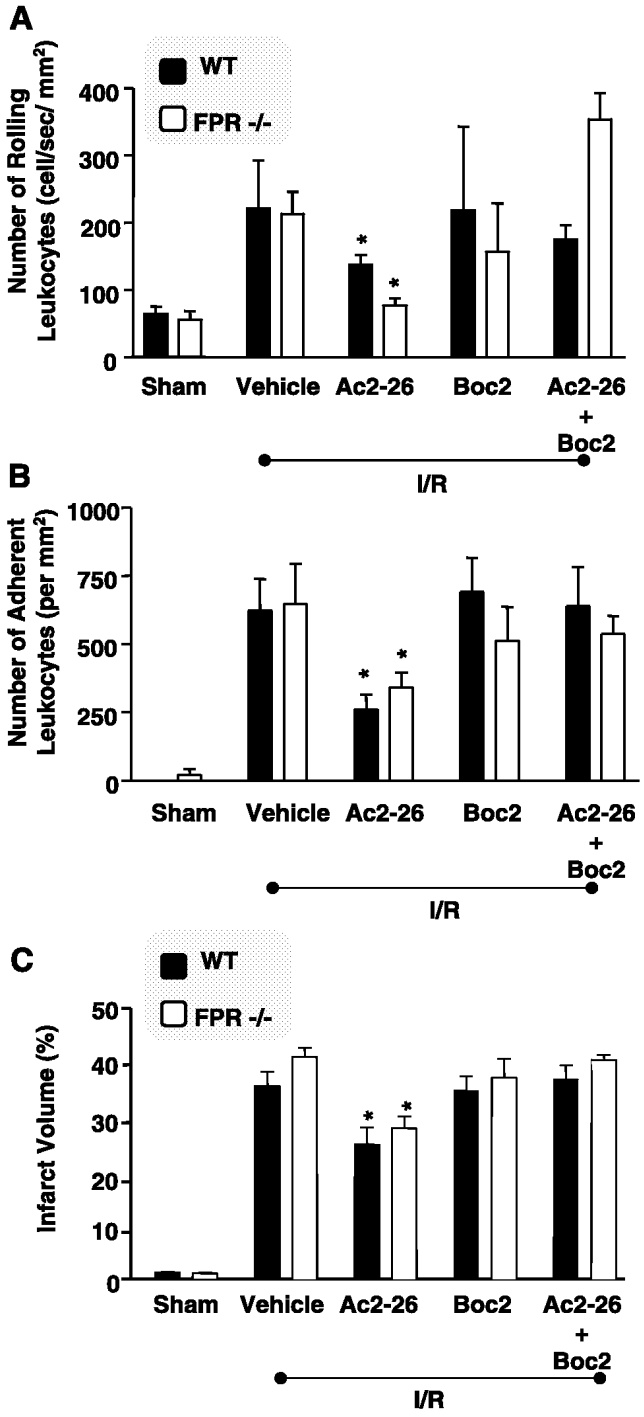

Figure 6. Peptide Ac2–26 protective actions in FPR null mice. C57BL/6 (WT) or FPR$^{-/-}$ mice were subjected to MCA I/R (1h) followed by 24 h reperfusion; peptide Ac2–26 was given three times at the dose of 100 μg per each injection as detailed in Materials and Methods. The leukocyte-endothelium interaction was quantified in terms of (*A*) cell rolling and (*B*) number of adherent cells. *C*) Infarct vol was also determined. Data are mean ± SEM of 6 mice per group. *$P <$ 0.05 *vs.* respective sham value.

30). The FPR family expands in the mouse, with at least seven receptors being identified so far (31). We have previously highlighted a functional role for mouse FPR in the tissue protection afforded by AnxA1 and peptide Ac2–26 (12). This complex family of receptors seem to mediate both pro- and antiinflammatory signals, a finding likely to be associated with the versatility of GPCRs and their ability of acquiring ligand-specific conformations (30, 32). We hypothesized that as the murine counterpart of human FPRL-1, FPR-rs2 [which is expressed by blood cells as well as by microglia, and transduces the proinflammatory and injurious actions of beta-amyloid protein as well as the protective actions of humanin (31, 33)], may be mediating the cerebroprotective effects of AnxA1 in our MCA I/R model. Thus, since there are no published data for peptide Ac2–26 binding to receptors of the mouse FPR family, we used HEK cells transfected with specific receptors (34) to demonstrate that peptide Ac2–26 binds to both FPR and FPR-rs2 with an approximate EC_{50} of 1 μM, in line with what reported for human FPR and FPRL-1 (14, 22) and rat FPRL-1 (25). Integration of these data with the *in vivo* results obtained with the FPR antagonist, Boc2 and gene deficient mice, we can indicate FPR-rs2 as the receptor mediating the cerebro-protective actions of peptide Ac2–26.

It is noteworthy that AnxA1 and peptide Ac2–26 share at least one receptor with other antiinflammatory mediators, lipoxin A_4 and aspirin-triggered epi-lipoxin A_4 (14), shown to exert organ protection in several experimental settings (35, 36). Whereas aspirin-triggered epi-lipoxin A_4 is produced by the concerted action of aspirin-acetylated COX-2 and 12- or 5-lipoxygenase (LOX), a new class of antiinflammatory lipids derived from other polyunsaturated fatty acids has been discovered by Serhan and colleagues (37): A derivative of docosahexaenoic acid, neuroprotectin D1, displays potent inhibitory effects on retinal pigment epithelium injury (38). Of interest, a recent study conducted with neural stem cells shows that lipoxins would also activate mouse FPR-rs2 (39), thus indicating that the receptor sharing properties between AnxA1, its peptides and these short-lived lipids are not restricted to FPR-rs1 (12).

Some of the data presented here, including local mediator generation and receptor involvement, point to the microglia as a second potential target for the effects of AnxA1. Myeloid cells, including tissue macrophages and lineage related microglia cells (40), express high AnxA1 levels. Recent work has shown how, on cell activation, this 37 kDa protein translocates on the cell surface to interact with a specific G-protein coupled receptor, termed FPRL-1 (14). FPRL-1, and its murine counterpart mFPR-rs2, are expressed on peripheral leukocytes (34, 41) and microglia cells (18, 31).

Local inflammatory responses were also generally augmented by AnxA1 deficiency. We investigated the effect of some key inflammatory markers, MCP-1, KC, and IL-1β. MCP-1 has been involved in the trafficking of mononuclear cells into the brain parenchyma after stroke (42), whereas KC is a chemotactic agent for neutrophils. The proinflammatory nature of IL-1β in several models of brain inflammation, including stroke, is well established (43, 44). It is plausible that microglia are at least contributory to generation of these proinflammatory mediators, strongly suggesting that endogenous AnxA1 might play a homeostatic role in this cell type, along the line of the studies conducted with the lineage relevant cells, the peripheral and the bone marrow derived macrophage (45, 46). There are yet no data in relation to microglia activation and AnxA1 gene deficiency, but these derivative data, coupled to the pharmacological effects of peptide Ac2–26 on microglia cell activation *in vitro* (47) and the augmented expression of the protein in this cell type in experimental models of injury (48, 49), would justify future studies. As said previously, microglia and blood cells express FPR-rs2 that is likely to mediate the cerebroprotective effects of peptide Ac2–26. In a preliminary experiment, minocycline was used to silence microglia noting that it markedly reduced leukocyte adhesion to pia mater venules (F.N.E.G. and M.P., unpublished). Clearly further investigation is needed.

In conclusion, the present results indicate the possibility that, as in the periphery (8, 9), multiple endogenous counter-regulatory pathways exert a tonic inhibitory role of cerebral molecular and cellular responses to injury. In line with current views on the role played by mediators of the innate protective response (50), we have shown here some of the properties of endogenous AnxA1 and how this profile could be exploited by bioactive peptides and associated receptors as possible new therapeutic targets. FJ

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